Editorial

From transcriptional noise to modulator of the TGFB1 pathway, a player in the development of chemoresistance: lncRNA-LET

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In order to win the battle against cancer, two hurdles need to be overcome; the development of drug resistance and the spread of cancer through metastasis. The article by Zhuang *et al.* (1) investigated the former in bladder cancer, whereby a very high incidence of death is due to a large percentage of patients developing resistance to chemotherapy despite their original early response. To date, the most tolerated chemotherapeutic used in treatment of this disease is a gemcitabine/cisplatin combination. Gemcitabine is a deoxycytidine analogue that causes cytotoxicity: blocking cell progression in the G1/S phase by attaching/inhibiting both active DNA and ribonucleotide reductase synthesis (2,3). In an attempt to unfold the multifaceted interaction between orchestrators and effectors in cancer stem cell (CSC) enrichment, Zhuang *et al.* set out to identify mechanisms underlying the development of chemoresistance in bladder cancer with the hypothesis that the transforming growth factor-β1 (TGF-β1) pathway regulates the long non-coding RNA (lncRNA)-LET (low expression in tumor)/NF90/miR-145 axis which in turn promotes gemcitabine resistance in bladder cancer. The current publication is one of few exploring the dynamic interplay between lncRNA and microRNA (miRNA) affecting bladder cancer pathophysiology.

Large scale transcriptome analyses of the mammalian genome have revealed that only a limited percentage codes for proteins while the majority is non-coding, including lncRNA. The study of the biological function of lncRNA is still young and their functional biological role is yet to be fully elucidated. LncRNA are >200 nucleotides in length and similar to miRNAs, are transcribed by RNA polymerase II and carry no coding, or low coding capability. Their diverse roles can be summarized as regulators of gene expression: in acting as transcriptional regulators through directly interacting with DNA thus modifying chromatin conformation, in an epigenetic role by modifying chromatin-complexes leading to regulation of promoter regions, as well as their interaction with miRNAs in a sponge-like fashion inhibiting them from interacting with their target mRNA. LncRNA are highly tissue and cancer specific, which present a great potential target for therapy and intervention once their functional role is completely identified and verified.

One postulation for the development of progressive drug resistance in bladder cancer is the activation of dormant CSCs. CSCs are a subpopulation of cells that possess the ability to self-renew and maintain tumor propagation. As CSCs divide they retain their self-renewal capacity and may also give rise to differentiated specialized progenitor cells (4,5). It is speculated that CSCs have specific properties that help develop a drug refractory state in a percentage of urothelial carcinoma patients. There have been many publications on the potential implication of CSCs in drug resistance, specifically in hematologic disorders, with few papers looking into their role in bladder cancer (6). The first challenging question addressed in the work by Zhuang and colleagues was to identify any differentially expressed lncRNAs in a population of gemcitabine treated mice. They reported that in the drug resistant group, lncRNA-LET was down-regulated, with an increase in CSCs markers

CK5 and CK14. Furthermore, in an effort to demonstrate tumor cells become resistance to gemcitabine treatment via CSCs, a sphere assay was performed showing cells in the gemcitabine treated group increased the number of spheres derived from xenografts, compared with control.

The cache of expression and functions of lncRNA-LET in cancers is slowly being uncovered. It has recently been shown to be down-regulated in many tumor types including lung adenocarcinoma, cervical, hepatocellular, colorectal and nasopharyngeal (7-10). In a meta-analysis by Liu *et al.*, it was shown that tumors with low lncRNA-LET expression showed higher incidence of lymph node metastasis and a poorer overall survival. In gastric cancer, lncRNA-LET expression was reported to be epigenetically silenced by alterations of EZH2 and HDAC3 in lncRNA-LET promoter regions under the regulation of lncRNA-DANCER. Its decreased expression in tissues leads to NF90 protein stabilization, which in turn increases hypoxia-induced tumor invasion capacity (7).

In an *in vitro* setting, the authors measured lncRNA-LET expression in three selected bladder cell lines representing TCC: T24, J82 and 5637. T24 and 5637 showed increased expression; therefore, two siRNAs were used for lncRNA-LET knock down. On the other hand, J82 expressed low levels of lncRNA-LET and forced expression was implemented. These alterations in expression levels were followed by assessment of $ALDH^{high}$ populations and protein levels of CSC basal stem markers (CD44, KLF4 and HMGA2). The authors showed an inverse proportional relationship between lncRNA-LET expression and the detection of $ALDH^{high}$ populations.

To explore the biological significance of lncRNA-LET in bladder cancer cells, the researchers established T24 and 5637 xenografts in nude mice treated with gemcitabine or control, which were used to illicit the role of lncRNA in driving an increase in the bladder CSC population. PCR array profiling of these xenografts for lncRNA expression was performed, revealing lncRNA-LET being the only lncRNA assayed that was more than two folds down-regulated in gemcitabine treated xenografts derived from both T24 and 5637 cells. To better understand the dysregulation of the TGF-β pathway and the role it plays in gemcitabine resistant xenografts, a TGF-β1 receptor kinase inhibitor (LY2157299) was administered alone or in combination with gemcitabine along with a third control group. Concomitant treatment with gemcitabine and LY2157299 was the most effective in tumor size reduction.

Bladder cancer is known to harbor a multitude of genetic mutations and signaling pathway abnormalities (11) including the TGF-β pathway. The TGF-β pathway regulates two important downstream cascades: as a key signaling route where epithelial-mesenchymal transformation (EMT) takes place, as well as, its role in miRNA regulation. During EMT, epithelial cells undergo modifications losing their epithelial markers such as E-cadherin and β-catenin, with an increased expression of N-cadherin, fibronectin and vimentin representing mesenchymal phenotypic markers. This ultimately augments tumor cell motility and migration capabilities. The EMT process contributes to the invasion cascade, leading to spread of cancer cells from the original site to distant metastatic locations, with a worse pathologic staging and prognosis. Finding strategies and therapies to deter EMT will prove to be a very valuable addition to cancer treatments.

To further investigate the interactive pathways, Zhuang *et al.* compared expression levels of several members of the TGF-β pathway between T24 gemcitabine treated and control xenografts. They reported increased expression of p-SMAD2 in the treated group. In order to validate an interconnection between down-regulated lncRNA-LET and the induction of the TGFBR1 super family mediated by SMAD2, the authors used computational analytical tools for functional analysis which identified a SMAD binding element linked to the promoter region of lncRNA-LET. This was validated by luciferase assay comparing wild-type LET and SMAD binding element deleted promoters.

Subsequently, they used limiting dilution assay to examine tumor self-renewal and regeneration capacity. Using lncRNA-LET stable knockdown established cells (shLET), it was shown that in depleted tumors, there was increased cancer incidence and tumor volumes. The reverse process was tested by forced overexpression of lncRNA-LET in T24 cells showing a decrease in CD44, KLF4 and HMGA2 phenotypic stem markers. Nude mice inoculated with the established lncRNA-LET overexpressing cells, were treated with gemcitabine. An increase in tumor latency occurred, with concomitant IHC data showing that CK5 and CK14, CSC markers, were highly induced in the gemcitabine mice versus the controls.

These data collectively demonstrate that lncRNA-LET is essential and sufficient for maintenance of urinary bladder cancer (UBC) stemness, whereas forced overexpression of lncRNA-LET could partially reverse

chemoresistance in UBC.

To investigate another layer of the oncogenic drivers contributing to this labyrinthine of interactions, the authors noted increased levels of the RNA binding protein NF90 in T24 and 5637 lnc-LET knockdown xenografts; whereas decreased expression of NF90 was observed in a J82 lncRNA-LET forced expression model. It was also determined that NF90 expression was manipulated by lncRNA-LET dictating the action of 26S proteasome system. The authors proposed that through the interaction between NF90 protein and lncRNA-LET, a feedback loop is created. Forced decreased expression of both lncRNA-LET and NF90 resulted in decrease CSC markers, and thus the ability of CSCs to revert gemcitabine lethality in bladder cancer cells.

To further elucidate this chemoresistance pathway the authors investigated miRNAs, an extensively studied subclass of non-coding RNA (each comprised of 19–25 nucleotides in length). To date, more than 2,500 miRNAs have been identified with a delineated role as critical regulators of post-transcriptional gene expression regulating protein coding within the cell (12). Many miRNAs have been implicated in carcinogenesis of the bladder. One of the main obstacles in cancer research is identification of the cellular function and extensive interaction between cellular complexes (mRNA, miRNA, and proteins) that are involved in the activation, inhibitory feedback loops, and degradation of lncRNA.

A complimentary role of NF90 is its regulation of miRNAs. In a multi-step analysis of miRNA expression by microarray and hierarchical clustering, the authors revealed NF90 negatively regulates miR-145. Next, they validated this finding by NF90 knockdown and measurement of miR-145 expression. When they measured the level of miR-145 expression in lncRNA-LET overexpressed cells it was upregulated. The authors report that collectively these data indicate that an lncRNA-LET/NF90/miR-145 axis exists in UBC cells. In addition, they utilized a reporter assay to confirm the direct interaction between miR-145 and the promoters of the cancer stem markers KLF4 and HMGA2.

This newly discovered axis was verified in a clinical setting using over 100 urologic cancer samples between tumor and normal tissue. A Kaplan-Meier survival analysis of their data set of urothelial bladder cancer patients showed that lower expression levels of lncRNA-LET and miR-145 were associated with a reduced survival rate, whereas TGF-β1 expression level, independently, had no clinical significance. Patients with a profile of TGF-$\beta1^{low}$/lncRNA-LET^{high}/miR-145^{high} showed an improved survival rate in comparison to those exhibiting TGF-$\beta1^{high\ or\ low}$/lncRNA-LET^{low}/miR-145^{low} profiles. Overall, the authors argue that a combined panel of TGF-$\beta1^{high}$/lncRNA-LET^{low}/miR-145^{low} signature in chemoresistant bladder cancer can be used as a prognostic clinical biomarker.

While few limitations have been noted in the current work, the dose-dependent effect of gemcitabine was not tackled. Additionally, factors within the tumor microenvironment, such as hypoxia, may have also contributed to enrichment of CSCs and induction of EMT markers. Hypoxia induced factors (e.g., HIF1) contribute to the stimulation of CSCs, and an investigation into their potential role is of interest to this question. Work by different groups showed that hypoxia induced histone deacetylase 3 repressed lncRNA-LET by reducing the histone acetylation-mediated modulation of its promoter region. In the article by Zhuang *et al.* there was no experimental data addressing if hypoxia and its effects, as partial players in CSC deployment, factored into their results. To date, there is no gold standard cell marker profile in urothelial tissue that is ubiquitously agreed upon to differentiate CSCs, progenitor and differentiated cells. In previous published literature, basal cell markers such as cytokeratin 5, 20 and 17 have been all specifically shown in bladder cancer, in addition to CD133, CD47, CD49 and KRT14. It would have been interesting to see such marker presentation in the lncRNA-LET knock down and forced expression cell lines. Lastly, it would have been interesting to see if lncRNA-RoR (regulator of reprogramming) was differentially expressed as it has been shown to function as a competing endogenous RNA (ceRNA) sponge for miR-145.

The author's work is an extensive effort in the quest to unravel the complex interactions of non-coding RNA. Additional validated research is needed to address the key role of epigenetic gene regulation, including this type of lncRNA-miRNA axis, as a driver for the preferential growth of CSCs and the scheme by which it affects the EMT pathway in increasing tumor stemness and chemoresistance. The possibility of manipulation of expression of lncRNAs such as lncRNA-LET in bladder cancer via technologies such as siRNA-silencing or modification/interception of the feed-back loop between them and miRNA offers novel possibilities in the treatment of chemoresistance.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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